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Novel mutations in *RASGRP2* encoding for CalDAG-GEFI abrogate Rap1 activation causing platelet dysfunction.

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Abstract

In addition to mutations in *ITG2B* or *ITGB3* genes causing defective $\alpha\text{IIb}\beta 3$ expression/function in Glanzmann's thrombasthenia patients, platelet dysfunction can be due to genetic variability in proteins mediating inside-out activation of $\alpha\text{IIb}\beta 3$. The *RASGRP2* gene is strongly expressed in platelets and neutrophils, where its encoded protein CalDAG-GEFI facilitates the activation of Rap1 and subsequent activation of integrins. We used next-generation (NGS) and whole exome sequencing (WES) to identify two novel function-disrupting mutations in *RASGRP2* accounting for the bleeding diathesis and platelet dysfunction in two unrelated families. Using a panel of 71 genes, we identified a homozygous change (c.1142C>T) in exon 10 of *RASGRP2* in a 9-year old child of Chinese origin (Family 1). This variant led to a p.Ser381Phe substitution in the CDC25 catalytic domain of CalDAG-GEFI. In two Spanish siblings from Family 2, WES identified a nonsense homozygous variation (c.337C>T) (p.Arg113X) in exon 5 of *RASGRP2*. CalDAG-GEFI expression was markedly reduced in platelets from all patients and, using a novel *in vitro* assay, the nucleotide exchange activity was found to be dramatically reduced in CalDAG-GEFI p.Ser381Phe. Platelets from homozygous patients-exhibited agonist-specific defects in $\alpha\text{IIb}\beta 3$ integrin activation and aggregation. In contrast, α - and δ -granule secretion, platelet spreading and clot retraction were not markedly affected. Integrin activation in the patients' neutrophils was also impaired. These patients are the first cases of a CalDAG-GEFI deficiency due to homozygous *RASGRP2* mutations that are linked to defects in both leukocyte and platelet integrin activation.

Key points:

- Novel function-disrupting mutations in CalDAG-GEFI, p.Ser381Phe and p.Arg113X, were identified in two unrelated families of different ethnic origin.
- Homozygous carriers of these mutations displayed a moderate bleeding diathesis and an impairment/delay in the platelet aggregation response to most agonists.

Introduction

One of the most severe inherited platelet disorders^{1,2} (IPDs) is Glanzmann's thrombasthenia (GT), which is caused by recessive mutations in either *ITG2B* or *ITGB3* leading to defects in $\alpha\text{IIb}\beta 3$ integrin activation and/or expression, and, consequently, severely impaired platelet aggregation in response to all platelet agonists^{3,4}. Impaired platelet aggregation and bleeding can also be caused by mutations in genes for signaling proteins critical in the inside-out activation of $\alpha\text{IIb}\beta 3$. *RASGRP2* was recently identified as one such gene affected in patients with a platelet function defect and a bleeding complication⁵. *RASGRP2* codes for the protein CalDAG-GEFI, a guanine nucleotide exchange factor for the small GTPase Rap1. In platelets, Rap1 activity is regulated by two independent yet synergistic signaling pathways⁶. A fast but reversible activation pathway mediated by an increase in the intracellular calcium ion concentration and the activation of CalDAG-GEFI. And a slow but sustained pathway that requires signaling by protein kinase C and the ADP receptor, P2Y₁₂, the target of anti-platelet drugs such as clopidogrel. While CalDAG-GEFI mediates GTP loading, i.e. the activation of Rap1, signaling by P2Y₁₂ leads to the inhibition of Rasa3, a GTPase-activating protein critical in the inactivation of Rap1⁷. In mice, genetic deletion of *Rasgrp2* did not affect embryonic development and survival. Adult mice lacking CalDAG-GEFI showed marked protection from experimental thrombosis and a significant impairment in hemostasis^{8,9}. Furthermore, these mice exhibited a mild defect in the adhesive function of neutrophils, another cell type that expresses significant levels of CalDAG-GEFI¹⁰. The studies by Canault et al.⁵ provided the first genetic evidence that (1) CalDAG-GEFI is also a critical regulator of integrin-mediated adhesion in human platelets, and (2) the molecular machinery controlling Rap1 activity in human platelets closely resembles that of murine platelets. No defects in neutrophil adhesion mediated by integrins were observed.

In this study, we report two pedigrees characterized by a bleeding tendency and a platelet dysfunction due to impaired $\alpha\text{IIb}\beta 3$ integrin activation. Neutrophils from homozygous patients also showed a defect in $\beta 2$ -integrin activation *in vitro*, but no relevant infections were reported. These cases carry novel mutations in *RASGRP2*, a nonsynonymous change c.1142C>T in Family 1 and a nonsense change c.337C>T in Family 2. Both mutations led to a marked reduction in CalDAG-GEFI expression. Thus, these patients are the first cases of

a CalDAG-GEFI deficiency due to homozygous *RASGRP2* mutations that are linked to defects in both leukocyte and platelet integrin activation.

Methods

Subjects, blood sampling and DNA collection

The study involved three index cases from two unrelated families of Chinese (Family 1) and Spanish origin (Family 2). Venous blood was drawn from each patient and a parallel healthy control into 7.5% K3 EDTA tubes (for blood counts and DNA isolation), and into buffered 0.105 M sodium citrate (for platelet and neutrophil function studies) using a 20-gauge needle. Samples were maintained at room temperature until processing. Genomic DNA from EDTA blood samples was isolated using a DNeasy blood and tissue kit, following the manufacturer's protocol (Qiagen, Hilden, Germany). DNA concentration was measured using a Qubit 2.0 fluorometer (Life Technologies, Carlsbad, CA).

This investigation followed the Helsinki Declaration and had approval by the Ethics Committee of the Hospital Reina Sofía (Murcia, Spain). All subjects provided written informed consent.

NGS gene panel

A clinical NGS panel was designed (Design Studio, Illumina, San Diego, CA) with probes targeting all exons, 3'UTR and flanking regions, of each of the 71 genes depicted in Supplemental Table 1. The NGS was performed on a MiSeq Instrument, running MiSeq Control Software and according to a Nextera sequencing design (Illumina). This NGS panel was applied in the index case of Family 1. In brief, fifty ng of the DNA was sequenced following Illumina's standardized protocol. DNA libraries were normalized to 4 nM and pooled in equal volumes. We used the 300-cycle reagent kit. Specifically, the sample was sequenced using paired 150 nt reads, multiplexing twelve, dual indexed samples per run. Each run performed in this study contained twenty-four samples. Minimum coverage per base was 100 reads. High sequence quality was based on Phred score of > 20, Quality > 20 and Read coverage > 30 at each position within the reads. Sequence data was mapped to the

revised Cambridge Reference Sequence using the MiSeq's integrated computer software platform (MiSeq Reporter, Illumina).

Whole exome sequencing (WES)

In two patients from Family 2 the sequencing of the whole exome was performed essentially as described¹¹. Following enrichment of coding regions and intron/exon boundaries with the SureSelect human AllExon 50Mb kit (Agilent Technologies) captured libraries were sequenced on the Illumina HiSeq 2500 (Illumina) with 100 bp paired-end reads. Bioinformatic analysis was carried out as in previous studies^{11,12}.

Sanger sequencing

Verification of the two mutations in the *RASGRP2* gene that were identified in these patients by NGS and WES, and their segregation in the pedigrees was achieved by standard Sanger sequencing with specific primers, using an ABI 3730 automated sequencer. The following primer pairs were designed using the ExonPrimer script (<http://ihg2.helmholtz-muenchen.de/ihg/ExonPrimer.html>):

- a) mutation C.1142 C>T [p.Ser381Phe]

RASGRP2 x 5F: GTCGACCTTTGGCCCTG

RASGRP2 x 5R: AGTGCTCCGGCAAACCTGG

- b) mutation C.337 C>T [p.Arg113]

RASGRP2 x 5F: GGTGGTTGTCTCAAGGGTCT

RASGRP2 x 5R: CGAGAGAGCAACATGACCCT

Sequences in the PCR products were analysed using MutationSurveyor software available from SoftGenetics (<http://www.softgenetics.com/mutationSurveyor.html>).

Platelet aggregation

Platelet rich plasma (PRP) was obtained by centrifugation of citrated whole blood at 150 x g for 10 min. Platelet poor plasma (PPP) was prepared by a further centrifugation step of the same tube at 1000 x g for 20 min. Light transmission aggregometry (LTA) was performed essentially as described³ using undiluted PRP in an Aggreco II aggregometer (Menarini Diagnostics, Florence, Italy). Time course changes in the maximal percentage of light transmission of PRP over baseline (PPP) were recorded for 300s upon addition of various

platelet agonists (1.5 mM arachidonic acid, 25 μ M Thrombin Receptor Activating Peptide (PAR1p), 2 and 10 μ g mL⁻¹ collagen, 10 μ M ADP, or 100 nM phorbol 12-myristate 13-acetate (PMA).

Platelet ¹⁴C-Serotonin release

Platelet rich plasma was adjusted with homologous PPP to a the final platelet count to 250 x 10⁹L⁻¹ and labelled with 1 μ M ¹⁴C-serotonin (5-OH-¹⁴C-labelled tryptamine [¹⁴C-serotonin]; GE Healthcare, Barcelona, Spain) for 45 min at 37°C as described¹³. Platelet activation/aggregation in the labelled PRP samples was performed as above with selected agonists and 1/6 volume of EDTA 0.05 M-formaldehyde 0.633 M was added to stop reactions. Then, cuvettes were centrifuged (12.000 x g, 2 min) to pellet platelets and ¹⁴C-serotonin released into the supernatant was quantified by a liquid scintillation counter Wallac 1409 (Wallac Oy, AG&G Company, Turku, Finland), and reported as percentage of the total radioactivity incorporated, after correction for background¹³.

Clot retraction

Clot retraction assays were performed essentially as described¹⁴. Briefly, PRP samples adjusted with homologous PPP to a the final platelet count to 225 x 10⁹L⁻¹, were mixed in aggregation cuvettes with Tyrodes-Hepes buffer also containing small amount of red-blood cells. Clot formation was induced at RT with thrombin (1 U mL⁻¹) and CaCl₂ (2 mM), a sealed glass pipette was set at the center of the cuvette and clot retraction was monitored over time.

Platelet flow cytometry

Platelet expression of major platelet membrane glycoproteins (GP) (GPIa, GPIb α , GPIIb [α IIb], GPIIIa [β 3]) was assessed by flow cytometry in citrated whole blood diluted 1:10 in saline buffer through a direct standard technique with appropriate labeled monoclonal antibodies in a FACScalibur platform (Becton Dickinson, San Jose, CA). For analysis of surface-expressed P-selectin (marker of α -granule release), and binding of fibrinogen (marker for activated α IIb β 3), diluted PRP (\approx 20 x 10⁹L⁻¹ platelets) was stimulated under static conditions (30 min at room temperature) with the desired agonist concentration in the

presence of both anti-CD62-PE antibody (BD Biosciences, Madrid, Spain) and fibrinogen-Alexa488 (Thermo Fisher, Madrid, Spain). Samples were then run in FACSCalibur flow cytometer (Becton Dickinson) and the fluorescence of positively stained cells was analysed using CellQuest software.

Platelet spreading

Washed platelets were prepared as described¹⁵. Coverslips were coated overnight in 100 µg mL⁻¹ fibrinogen (Enzyme Research Lab, South Bend, IN) in sterile PBS at 4°C. The coverslips were blocked in denatured, sterile filtered 5mg/ml BSA (First Link UK Ltd, Birmingham, UK) in PBS. Three hundred µl of washed platelets, adjusted to at 20 x 10⁹ L⁻¹, were added to each well and allowed to spread for 45 minutes at 37°C. Spread platelets were washed in PBS and fixed at room temperature in 4% PFA for 5 minutes. Images were taken using a DM IRE2 Leica inverted microscope, SP2 confocal system running Leica Confocal Software Version 2.61 Build 1537. Platelet spreading assays were imaged using reflectance microscopy using the 488 nm line of an Argon-Ion laser 457-514 nm with an HCX Plan Apo Ibd.BL 63x NA 1.4, Olympus objective. In order to calculate platelet spread area, 10 platelets from 10 different fields of view (FOV) (100 platelets per sample) were outlined using the ROI tool in ImageJ (1.48V). The area measurements were then logged. Statistical significance was assessed by one-way ANOVA.

Immunoblotting

Total lysates from washed human platelets were separated by SDS–polyacrylamide gel electrophoresis on 4%–20% gradient gels and transferred to polyvinylidene fluoride membranes (Millipore). Standard Western blotting procedures were used. RAP1 (α-RAP1 clone 121, Santa Cruz Biotechnology, Dallas, TX) and CalDAG-GEFI (polyclonal rabbit α-RasGRP2, ThermoFisher Scientific, Waltham, MA) proteins were detected using IRDye 800-conjugated goat anti-rabbit secondary antibodies (Li-Cor Biosystems, Lincoln, NE). RASA3 (polyclonal goat α-GAP1-InsP₄BP (RASA3), Santa Cruz Biotechnology) and β-actin (α-β-actin clone AC-15, Sigma-Aldrich, St. Louis, MO) proteins were detected using IRDye 680-conjugated donkey anti-goat and goat anti-mouse secondary antibodies (Li-Cor Biosystems), respectively, and visualized with the Odyssey Infrared Imaging System (Li-Cor Biosystems).

CalDAG-GEFI expression and purification

Human CalDAG-GEFI and Rap1B proteins were overexpressed in *E. coli* and purified using a combination of immobilized metal affinity and size exclusion chromatography. Purified proteins were homogenous based on SDS-PAGE. Wild-type and mutant forms of CalDAG-GEFI were expressed as truncations lacking 58 N-terminal residues predicted to be unstructured [CalDAG-GEFI (1-551)], while Rap1B was truncated at Cys181 and this residue mutated to serine [Rap1B (C181S)]. CalDAG-GEFI harboring S381F was not submitted to size exclusion chromatography due to protein instability.

Nucleotide exchange activity assay

Purified proteins were used to determine the GEF activity towards Rap1B of wild-type and mutant CalDAG-GEFI. Nucleotide exchange on Rap1B was monitored using Bodipy FL GDP, a fluorescent analog of GDP. The fluorescence of Bodipy FL GDP is quenched in solution and increases upon binding to Rap1B. To determine the GEF activity of CalDAG-GEFI mutants, purified Rap1B (1 μ M) was added to exchange buffer (20 mM Tris pH 7.5, 150 mM NaCl, 2 mM MgCl₂, 0.08% NP-40, 5% (v/v) glycerol, and 1 mM DTT) containing 1 μ M GDP-BODIPY (Thermo Fisher), both in the absence or presence of 400 nM wild type or mutant CalDAG-GEFI. Fluorescence data were collected on a Pherastar microplate reader (BMG LABTECH, Ortenberg, Germany) with λ_{ex} = 485 nm, λ_{em} = 520 nm and 2 nm slits.

Leukocyte studies

Citrated whole blood samples containing 1×10^6 WBCs were resuspended in ice-cold hypotonic NaCl solution (0.2%) for 30 s, after which hypertonic (1.6%) NaCl solution (v:v) was added to restore isotonicity and cells were pelleted (5 min, 350 x g at 10°C). The lysis step was repeated once, and WBCs were resuspended in saline. To analyze surface glycoprotein expression, monoclonal antibodies for membrane glycoproteins CD11a and CD11b were used (Becton Dickinson). β_2 integrin activation in stimulated neutrophils was analyzed by the ability of these cells to bind soluble fibrinogen or the conformation specific-antibody m24 (Abcam, Cambridge, MA); to analyze granule release, cells were stained with anti-CD11b Mac1 antibody (Becton Dickinson). Blood leukocytes were incubated with 60 μ g mL⁻¹ Alexa Fluor 488-fibrinogen (Thermo Fisher) and PerCP-anti CD45 (Becton Dickinson), PE-anti-Mac1 and PerCP-anti CD45, or with 10 μ g mL⁻¹ m24. Saline, PMA, N-formyl-methionine-leucine-

phenylalanine (fMLP), or manganese chloride (Mn^{2+}) were added during this phase, for 30 min at room temperature. Cells were washed, and either resuspended in saline, or tubes containing m24 were further incubated with a PerCP-anti mouse IgG1 (Santa Cruz Biotechnology) for 30 min, washed again, and resuspended in saline.

To analyze $\beta 1$ integrin expression, leukocytes were incubated with soluble VCAM-1/Fc chimera protein (R&D Systems, Minneapolis, MN), washed and stained with a FITC-conjugated antibody to human IgG (Sigma-Aldrich, Madrid, Spain). Flow cytometry was conducted immediately after the final wash.

A FACSCalibur flow cytometer (Becton Dickinson) and CellQuest and Paint-A-Gate softwares were used for analysis. Analysis of leukocyte subpopulations was performed on the basis of a CD45/side scatter (fibrinogen binding, CD11a and Mac-1 expression) or light scatter (m24 and VCAM-1 binding) gating procedure.

Statistical analysis

All functional assays were performed with samples from three patients and three healthy controls. Due to the limited sample number, statistical comparisons could not be performed.

Results

Patient general information

In this study we have characterized three patients from two unrelated families. The proband in Family 1 (P1-Family 1) is a 9-year-old Chinese child suffering from severe epistaxis requiring hospitalization and medical intervention, including nasal packing, tranexamic acid-treatment and transfusion of red blood cells and platelets. In the second family the index-case (P1-Family 2) is a 55-year-old Spanish woman, who has a lifelong history of spontaneous bruising, petechiae, epistaxis, gingival and gastrointestinal bleeding. Minor bleeding has been treated mainly with antifibrinolytic drugs. Menorrhagia required oral contraceptives and iron therapy. Her two childbirths, dental extractions and minor surgical procedures have been successfully managed with prophylactic platelet transfusions. Her 46-year-old brother, second proband in this family (P2-Family 2), also suffered from epistaxis, gingival and digestive bleeding in childhood requiring platelet transfusion. In adulthood, he

has experienced few bleeding episodes, but minor surgical procedures (colonoscopy and dental extractions) required the use of prophylactic desmopressin and antifibrinolytic drugs. In addition to their bleeding symptoms, these three patients displayed, on repeated testing, a normal platelet count and volume and mild anaemia (Table 1). Blood smears, leukocyte count and differential, markers of liver and kidney function, prothrombin time, activated partial thromboplastin time, fibrinogen and von Willebrand factor levels, were within normal ranges. Our patients did not display overt immune defects, or susceptibility to bacterial infections other than P2-Family 2 suffering from ulcerative colitis.

Platelet function studies

A clinical suspicion of inherited platelet disorder was established in these patients due to the prolonged bleeding time, extended PFA-100® closure times (Table 1), and the severely reduced platelet aggregation in response to ADP and low dose collagen (Figure 1A). The expression level of major adhesive glycoproteins including GPIb/IX, GPIa, and GPVI, was normal (not shown). Glanzmann's thrombasthenia was ruled out for the three patients as (i) the surface expression of $\alpha\text{IIb}\beta\text{3}$ was not affected (not shown), and (ii) the platelet aggregation was normal, or minimally impaired, in response to high concentrations of protease-activated receptor 1 agonist peptide (PAR1p), collagen, arachidonic acid, or PMA (Figure 1A). Compared to controls, the lag time, i.e. the time required to reach 10% aggregation, was markedly increased in platelets from patients stimulated with 10 $\mu\text{g/mL}$ collagen (0.93 ± 0.27 vs. 0.55 ± 0.07 min), 1.6 mM arachidonic acid (0.62 ± 0.12 vs. 0.24 ± 0.01 min) or 10 μM ADP (0.43 ± 0.11 vs. 0.18 ± 0.09 min) (all mean \pm SD; $n = 3$). No delay in the aggregation response to PMA or PAR1p was observed in the patient's platelets. Consistent with the delay in aggregation, platelets from the three patients exhibited markedly impaired fibrinogen binding upon stimulation with most agonists (ADP, PAR1p, PAR4p, CRP) (Figure 1B). In contrast, the patients' platelets showed only minor defects in agonist-induced α - or δ - granule secretion (Supplemental Figures 1A and 1B), clot retraction (Supplemental Figure 2), and spreading on fibrinogen (Supplemental Figure 3).

Identification of deleterious mutations in RASGP2.

Aiming to identify the molecular defects responsible for the bleeding phenotype in these patients, DNA from the Chinese boy and the Spanish siblings was analyzed by a novel NGS

panel covering 71-genes known or suspected to play a relevant role in IPDs (Supplemental Table 1) and whole exome sequencing (WES), respectively. As shown in Figure 2A, these molecular analyses identified novel homozygous changes in the *RASGRP2* gene in the index cases: i) a nonsynonymous change c.1142C>T (p.Ser381Phe) in exon 10 in Family 1; ii) a nonsense change c.337C>T (p.Arg113X) in exon 5 in Family 2. Sanger sequencing confirmed the presence of these mutations in the probands and their inheritance from the consanguineous parents (Figure 3). CalDAG-GEFI, the *RASGRP2* encoded protein, contains an N-terminal catalytic region, comprising a Ras exchange motif (REM) and a CDC25 domain that provides GEF activity, and a C-terminal regulatory region with two Ca²⁺ binding EF hand domains and a C1-like domain¹⁶ (Figure 2B). The nonsense c.337C>T (p.Arg113X) variant is expected to truncate CalDAG-GEFI synthesis at the level of the REM motif, while the p.Ser381Phe substitution is located at the C-terminal end of the CDC25 catalytic domain, and it is predicted by PolyPhen (<http://genetics.bwh.harvard.edu/pph2/>) to be “probably damaging” with a score of 1.0. The p.Ser381Phe variant is reported with an allelic frequency of 8.47×10^{-6} in the Exome Aggregation Consortium database (ExAC) (<http://exac.broadinstitute.org>), and it is unreported in the 1000 genomes project database (<http://browser.1000genomes.org>). The p.Arg113X is reported as somatic mutation in the COSMIC project (<http://cancer.sanger.ac.uk>) (All database accessed on 05/10/2016).

Novel *RASGRP2* mutations impair CalDAG-GEFI expression and function.

Immunoblotting analysis demonstrated that CalDAG-GEFI expression was markedly reduced in platelets from index cases homozygous for p.Ser381Phe or p.Arg113X and moderately decreased in platelets from available heterozygous relatives. In contrast, expression of Rap1 itself and Rasa3, a main Rap1-GAP in platelets⁷, was comparable in platelets from affected individuals and healthy volunteers (Figure 4). To evaluate whether the Ser381Phe substitution also impairs the GEF activity of CalDAG-GEFI, we purified the wild-type and mutant protein and subjected them to a novel, fluorescence-based assay to monitor nucleotide exchange on purified Rap1B. In this assay, nucleotide exchange on Rap1B is monitored with the help of Bodipy FL GDP, a fluorescent analog of GDP that is quenched in solution but shows increased fluorescence upon binding to Rap1B. As shown in Figure 5A, loading of Rap1 with Bodipy FL GDP occurred very slowly, unless it was catalyzed by the addition of CalDAG-GEFI. Interestingly, large amounts of CalDAG-GEFI (Ser381Phe), but not

CalDAG-GEFI (wild-type), precipitated during the purification process, suggesting altered stability of the patient protein. Non-aggregated CalDAG-GEFI (Ser381Phe) protein showed strongly reduced GEF activity when added to Rap1B at the same concentration as CalDAG-GEFI (wild-type). The reduction in the catalytic activity was similar to that observed with purified CalDAG-GEFI bearing a Gly248Trp substitution, the only other known CalDAG-GEFI mutation in humans⁵ (Figure 5A). Noteworthy, while Gly248 is positioned in the interface between the CDC25 domain and Rap1⁵, Ser381 localizes to an adjacent region (Figure 5B). Based on structural modelling, we propose that the p.Ser381Phe substitution clashes with Leu263, Ser305 and Leu309 and thus causes a conformational change in CalDAG-GEFI which affects both protein stability and nucleotide exchange activity.

Neutrophil function studies.

Studies in mutant mice identified a role for CalDAG-GEFI in neutrophil integrin affinity modulation and adhesion¹⁰. We thus investigated whether neutrophil function was also affected in homozygous carriers of the CalDAG-GEFI S381F and R113X mutations (Figure 6). Compared to controls, neutrophils from homozygous patients showed normal $\beta 2$ integrin and reduced $\beta 1$ integrin expression (Supplemental Table 2). $\beta 2$ integrin activation was impaired in stimulated neutrophils from homozygous RASGRP2 patients (Figure 6A and 6B). Thus, when activated with fMLP, which causes inside-out activation, or by agents such as Mn^{2+} that increase integrin affinity by direct and indirect (signaling) effects¹⁷, patient neutrophils displayed a markedly impaired ability to bind fibrinogen (Figure 6A). Consistently, the binding of an antibody detecting the active conformation of LFA-1 (m24) was also reduced in Mn^{2+} activated patient neutrophils when compared to controls (Figure 6B). In contrast, granule secretion was only minimally affected in neutrophils from homozygous patients (Figure 6C).

Discussion

We here report two families with a bleeding diathesis and platelet dysfunction, resulting from novel mutations in *RASGRP2* that affect the expression and/or function of CalDAG-GEFI. This defect causes a markedly impaired aggregation response to low concentrations of certain agonists, most prominently ADP and collagen, and a delay in aggregation, even when

stimulated with high concentrations of most agonists. Our findings, together with an earlier study⁵, strengthen the molecular heterogeneity of *RASGRP2* as a cause of inherited platelet disorder associated with impaired $\alpha\text{IIb}\beta 3$ integrin activation.

In addition to the defect in platelet $\alpha\text{IIb}\beta 3$ activation, we observed alterations in the expression or function of $\beta 1$ and $\beta 2$ integrins in neutrophils. Neutrophils from homozygous patients exhibited reduced surface expression of $\beta 1$ integrins, a finding that is in accordance with results reported in *Rasgrp2* knock-out mice¹⁰. Furthermore, patient neutrophils showed defects in agonist-induced inside-out activation of $\beta 2$ integrins, confirming that CalDAG-GEFI is critical for the affinity modulation various integrin subfamilies^{10,18}. The fact that fibrinogen binding to $\beta 2$ integrins was also impaired in patient neutrophils stimulated with Mn^{2+} supports the concept that Rap1 is involved in both inside-out and out-side-in signaling, as previously suggested¹⁹. Interestingly, recent studies by Canault et al. did not reveal any defects in integrin-mediated adhesion of neutrophils from patients with a loss-of-function mutation in *RASGRP2*⁵. As discussed by the authors, the absence of an adhesion defect in CalDAG-GEFI (pG248W) neutrophils may be explained by the fact these cells express normal level of mutant protein, i.e. that this mutation may spare structural domains that facilitate leukocyte adhesive functions. Further studies will be required to test this hypothesis.

Mice lacking CalDAG-GEFI are characterized by a very strong protection from experimental thrombosis, a moderate deficit in hemostasis, and a very mild immunodeficiency^{6,8-10}. Including the patients described in this paper, there are now a total of six patients (4 males, 2 females) with three distinct mutations in *RASGRP2*. All of the patients are characterized by impaired platelet aggregation to select agonists and markedly prolonged bleeding times. They all exhibit lifelong bleeding complications, including spontaneous bruising, petechiae, epistaxis, gingival and gastrointestinal bleeding. Interestingly, these bleeding complications seem to be less severe in adults, such as the 46-year old proband in family 2 who has experienced few bleeding episodes during adulthood. None of the reported patients showed clinical signs of immunodeficiency. Due to the small number of patients, it is not possible to evaluate if mutations in *RASGRP2* also lead to protection in humans from thrombotic complications associated with cardiovascular disease. This would seem likely, however, given the critical role of CalDAG-GEFI in platelet adhesion at high shear stress conditions and the fact that the Rap1 signaling response in human platelets is very similar to that in murine platelets.

In summary, we here report two novel mutations in RASGRP2 that lead to partially impaired integrin activation in platelets and neutrophils. Homozygous patients display a life-long, bleeding tendency, but they do not show signs of immunodeficiency. Only with the availability of increased numbers of patients, will it become clear whether they are protected against thrombotic disease.

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Authorship Contributions

MLL, SPW, WB and JR, designed the research and wrote the paper. GI, RAP and ARC provided patient samples and clinical data. JR, MLL, FF, and SF carried out the platelet phenotyping. AC, DSP, JR and WB carried protein analysis and cell model. JMB, JRGP, JMHR performed the NGS gene panel. NM and BJ did WES and Sanger sequencing. All authors critically reviewed and approved final version of the paper.

Disclosure of Conflicts of Interest

The authors declare no conflict of interest.

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Legend to Figures

Figure 1. Platelet aggregation and integrin activation is markedly impaired in index cases with lifelong bleeding diathesis from two unrelated families.

A) Platelet aggregation in response to the indicated platelet agonists was evaluated in unadjusted platelet-rich plasma from index cases (P) and a healthy and unrelated control (C). B) Platelets from index cases and healthy and unrelated controls (controls) (combined data from three subjects), were stimulated under static conditions (30 min at RT) with agonist (5 and 25 μ M ADP [ADP-5 & ADP-25], 25 μ M PAR1 peptide [PAR1p], 250 μ M PAR4 peptide [PAR4p], 2 and 10 μ g mL⁻¹ collagen-related-peptide [CRP-2 & CRP-10], 2 μ g mL⁻¹ convulxin [Cvx], and 100 nM Phorbol 12-myristate 13-acetate [PMA]) in the presence of fibrinogen-Alexa 488. The samples were evaluated by flow cytometry and the median fluorescence intensity [MFI] for Fibrinogen-A488 binding is shown.

Figure 2: Localization of the novel c.1142C>T (p.S381F) and c.337C>T (p.R113X) mutations within the *RASGRP2* sequence and the encoded protein CalDAG-GEFI.

DNA from index cases was analysed by next generation sequencing or whole exome sequencing and novel mutations in *RASGRP2* were identified. A) Localization of the novel c.1142C>T (p.S381F; Family 1) and c.337C>T (p.R113X; Family 2) mutations within the *RASGRP2* sequence. B) Schematic representation for CalDAG-GEFI showing the different domains: Ras exchanger motif (REM), catalytic domain (Cdc25), calcium-binding EF hands (EF) and C1-like domain (unknown function). The positions of the recently reported G248W mutation⁵ and the novel p.R113X and p.S381F mutations within the REM and Cdc25 domains are shown.

Figure 3: Family pedigrees and bleeding scores in two unrelated families carrying novel mutations S381F and R113X in CalDAG-GEFI.

The index cases in each family are indicated with black arrows. Bleeding in patients and available family members was evaluated and scored (BS) using the ISTH Bleeding Assessment tool²⁰. Filled and semi-filled black symbols indicate homozygosity and heterozygosity for the correspondent mutation in *RASGRP2*. Other family members (white symbols) were not-available for study.

Figure 4. Novel mutations R113X and S381F severely affect the expression of CalDAG-GEFI.

Immunoblot analysis for CalDAG-GEFI (CDGI), Rasa3, Rap1 and β -actin in platelet lysates from carriers of the indicated mutations in CalDAG-GEFI. Left: p.S381F (P1 homozygous; P2, P3, P4 heterozygous); Right: p.S381F mutation (P1 and P2 homozygous; P3 heterozygous). Protein expression in lysates from healthy and unrelated controls (C) analyzed in parallel is also shown.

Figure 5. The S381F substitution alters CalDAG-GEFI structure and markedly impairs its nucleotide exchange activity.

A) Bodipy fluorescence-based assay to monitor nucleotide exchange on purified Rap1B. Black arrow indicates where wild-type (green) or S381F (red) or G248W (blue) CalDAG-GEFI was added. The increase in fluorescence intensity, a measure of nucleotide exchange, over time is shown. B) A structural model of the S381F substitution using the mutagenesis feature in pymol. This model was built based on the crystal structure of CalDAG-GEFI²¹ and represents phenylalanine substituted for serine at position 430, the homologous position of serine 381 of CalDAG-GEFI. Based on this model, there are 3 residues that clash with the phenylalanine substitution at S430: Leucine 263, Serine 305, and Leucine 309.

Figure 6: Impaired integrin activation in leukocytes from homozygous carriers of the CalDAG-GEFI S381F and R113X mutations.

A,B) β 2 integrin activation. Neutrophils were kept resting or stimulated with the indicated agonists in the presence of Alexa Fluor 488-fibrinogen (A) or m24 antibody (B) to determine the activation state of α M β 2 and α L β 2, respectively. C) Granule secretion. Neutrophils were kept resting or stimulated with fMLP (1 mM) or PMA (100 nM) in the presence of a PE-labeled antibody to CD11b (Mac-1). Results are expressed as the fold increase in median fluorescence intensity in stimulated vs. unstimulated cells. Values are mean \pm SEM from data obtained in the three homozygous patients (black bars) and three healthy controls (gray bars).

Table 1. Blood parameters and clinical bleeding symptoms in homozygous carriers of the novel mutations S381F and R113X in CalDAG-GEFI

		P1-S381F	P1-R113X	P2-R113X
WBC ($\times 10^9/L$)		7.2 \pm 1.2	8.0 \pm 0.2	7.9 \pm 1.1
RBC ($\times 10^{12}/L$)		4.8 \pm 0.4	3.9 \pm 0.1	4.6 \pm 0.1
Hb (g/dL)		11.4 \pm 1.9	10.9 \pm 0.6	12.5 \pm 0.5
Ht (%)		37.7 \pm 8.9	32.9 \pm 2.5	38.5 \pm 0.5
Platelets ($\times 10^9/L$)		351.5 \pm 84.1	182.0 \pm 9.9	246.0 \pm 22.6
MPV (fL)		8.5 \pm 0.9	10.1 \pm 2.2	10.0 \pm 3.0
PFA-100 CT (s)	Collagen/Epinephrine	>300	>300	>300
	Collagen/ADP	>300	244	275
Location of bleeding and score	Epistaxis	4	4	4
	Cutaneous symptoms	1	2	-
	Minor wounds	1	-	2
	Oral cavity bleeding	1	-	-
	Gastrointestinal bleeding		4	1
	Final Bleeding score	7	10	7

Values are mean \pm SD from two different complete blood counts separated at least two years. *Abbreviations:* WBC: white blood cells; RBC: red blood cells; Hb: Hemoglobin; Ht: Hematocrit; MPV: Mean platelet volume; CT: closure time. The Table reflects the clinical significant bleeding for each symptom, grading the bleeding severity from 0 (absence of symptoms) to 4. The final bleeding score was generated by summing the severity of all bleeding symptoms reported by the patient²¹